AN ANALYSIS OF THE AGGREGATION STAGE IN THE DEVELOPMENT OF THE SLIME MOLDS, DICTYOSTELIACEAE. II. AGGREGATIVE CENTER FORMATION BY MIXTURES OF DICTYOSTELIUM DISCOIDEUM WILD TYPE AND AGGREGATELESS VARIANTS[1]

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In the development of the Dictyosteliaceae, aggregation begins when the population has reached the stationary growth phase. The individual myxamoebae, previously distributed at random, become elongated and radially oriented. They move in toward the centers of orientation and are crowded together in ramified streams as they do so. Mounds of cells are built up in this fashion and acquire the characteristic form of the pseudoplasmodium. Ultimately, the pseudoplasmodia give rise to organized, multicellular fruiting structures (Bonner, 1944).

Several features of this phenomenon have been investigated. Evidence obtained by Bonner (1947) supports the conclusion that aggregation is the result of a chemotactic response on the part of outlying cells to the production of a diffusible substance by individuals at or near the aggregative center. Study of interspecific and intergeneric cell mixtures (Raper, 1940) has indicated that not all species can enter communal aggregates and suggests that the aggregative response is specific.

The number of aggregative centers that can be formed by the myxamoebae is a function of both the population number and density (Sussman and Noël, 1952). Where the population density does not limit center formation, the number of centers produced is proportional to the number of cells. For *Dictyostelium discoideum* strain Nc-4, the number of centers/cell was found to be 4.73×10^{-4}, corresponding to a distribution of one center-initiating agency among approximately 2100 cells. For *D. purpureum* strain V-1, the value was 3.3×10^{-3}, equivalent to one center-forming agency among 300 cells. Examination of the distribution of center-forming capacity among small, replicate population samples revealed that under the conditions employed only a small proportion of the population possesses the ability to initiate the aggregative process.

One pertinent question, which could not be answered by study of the wild type populations exclusively, is concerned with whether or not single cells act as initiators of aggregation. The reasons for this limitation are given in another part of this paper. In order to settle the question, an analysis has been made of aggregations carried out by mixtures of wild type myxamoebae and aggregateless variants which are capable of responding to the aggregative stimulus but not of producing it. The results demonstrate the single cells do indeed initiate aggregation. Several other aspects of these mixed aggregations have been investigated and are described.

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Methods

A. Organisms and growth conditions

The strains used in this investigation were: *Dictyostelium discoideum* strain Nc-4 and two aggregateless variants of this stock, Nc-4 agg-53 and Nc-4 agg-53A. These were routinely grown in association with *Aerobacter aerogenes* on an agar medium whose composition has been given elsewhere (Sussman, 1951).

The technique of clonal plating, used in certain of the experiments reported here, has been previously described (Sussman, 1951). It is similar to the "spread plate" technique employed in the past for bacteriophage (Delbrück, 1946). The plaques of myxamoebae appear within the layer of bacterial growth after 3.5 days incubation at 22° C. During the following 24 hours, fruiting structures appear in the plaques.

B. Preparation of cells for aggregation experiments

44- to 50-hour plates containing myxamoebae in the stationary growth phase were harvested with cold, distilled water. The suspensions were spun in an International refrigerated centrifuge for 5 minutes at 1000 rpm in order to separate the myxamoebae from the remaining bacteria. After three more washes, the cells were suspended in Bonner's salt solution (Bonner, 1947). At this time, direct cell counts of the suspensions were made in the Levy chamber on 6 to 8 replicates. The variances encountered were satisfactorily small.

C. Deposition of cells on aggregation plates

The aggregation experiments were carried out on a washed agar-distilled water substratum, hereafter denoted as "minimal agar." This was prepared by washing 25 g. of Difco agar in 6 liters distilled water on a Buchner table-top filter. After washing, the agar was suspended in one liter of water and autoclaved. Plates were poured from this medium.

When aliquots of cell suspensions, ranging in volume from 0.01 to 0.2 cc., are placed on minimal agar, the cells are distributed homogeneously over a surface area whose size is a function of the volume delivered. In this manner, both the population number and density may be controlled effectively and the myxamoebae are found to aggregate and produce normal fruiting bodies.

Experiments to be described indicate that when cells are prepared by the procedure given and are deposited on minimal agar, no appreciable increase in cell number occurs after as much as 48 hours incubation. It must be noted, however, that the separation of myxamoebae from occluded bacteria is occasionally unsatisfactory. With such preparations, no trouble is encountered where the population on the minimal agar plates is very dense since the amoebae ingest the bacteria almost immediately. But in aliquots where the population density is not great, some bacteria escape ingestion long enough to form microcolonies. When these are finally entered by the myxamoebae, substantial proliferation ensues. This difficulty can be avoided almost completely by careful washing of cells and of the agar used in the minimal medium.

Results

A. Descriptions of the aggregateless variants

Two variants were employed in this investigation. The first of these, Nc-4 agg-53, was obtained by irradiation of the wild type with UV. The techniques used for its isolation as well as for the collection of a series of stocks displaying a wide spectrum of aberrant developmental patterns will be described elsewhere (Sussman and Sussman, in preparation).

The plaques produced by agg-53 are easily distinguishable from those of the wild type. The latter forms a spreading plaque with an irregular outline and diffuse periphery. Normal fruiting structures appear within its confines. In contrast, the plaque formed by agg-53 is much smaller, circular and has a very sharply defined periphery. A circular slime deposition or series of concentric depositions always appears, giving each plaque the appearance of a bull's eye. No signs of aggregation or the erection of fruiting structures are observable. Spores are not produced.

During serial passage of agg-53, a plaque type variant was found to arise occasionally. Upon sub-culture, it bred true and was designated Nc-4 agg-53A. The plaque produced by this variant spreads and has an irregular outline like that of the wild type. The interior of the plaque remains perfectly smooth. No signs of aggregation or the erection of fruiting structures are seen. Spores are not formed.

In the course of many serial passages, neither of the variants has been seen to produce a reverted or mosaic clone. However, this extreme stability is by no means true of all aggregateless stocks thus far isolated.

The question of whether or not the strains represent genic modifications cannot be answered since, at present, no system of recombination analysis is available for use. Arguments not based on segregation data are neither necessary nor sufficient for the distinction to be drawn and therefore will not be considered at this time.

It should be noted that Pfützner-Eckert (1950) has reported the isolation of a strain of *D. mucoroides* which is incapable of forming fruiting structures. From this author's description, however, it appears that the cells can perform some of the activities associated with aggregation and, under some conditions, even form small pseudoplasmodia. We have isolated similar varieties. Such cells when washed and distributed on minimal agar generally are capable of aggregating and producing normal mature fruiting structures although usually not in as great a number as the wild type produces. The inability of such cells to do the same on the growth plates is a problem which has not as yet been attacked.

B. The formation of aggregative centers by mixtures of Nc-4 wild type and the variants

A previous investigation (Sussman and Noël, 1952) has demonstrated that the number of aggregative centers produced by Nc-4 wild type cells is a function not only of the number of cells present but also of the population density. When the latter is less than approximately 100 cells/mm.^{2}, no centers are formed. As the density is raised (while the number of individuals is kept constant), the number of centers increases until at a density of about 200 cells/mm.^{2} a maximal number of centers is produced. This circumstance made it possible to determine if the ag-

gregateless individuals could respond to the stimulus imposed by wild type where the population density of the wild type was so low that, alone, they could not form centers.

Suspensions of washed Nc-4 wild type and agg-53, prepared according to the procedure previously described, were counted in the Levy hemocytometer and appropriate dilutions were made. From these, mixtures were arranged such that they contained a constant number of Nc-4 cells but varying numbers of aggregateless individuals. For example, in one experiment the mixtures all contained 5.0×10^5 cells/cc. of wild type while the numbers of agg-53 cells/cc. ranged from 5.0×10^5 to 6.0×10^7. Replicate drops of 0.01 cc. volume were distributed on

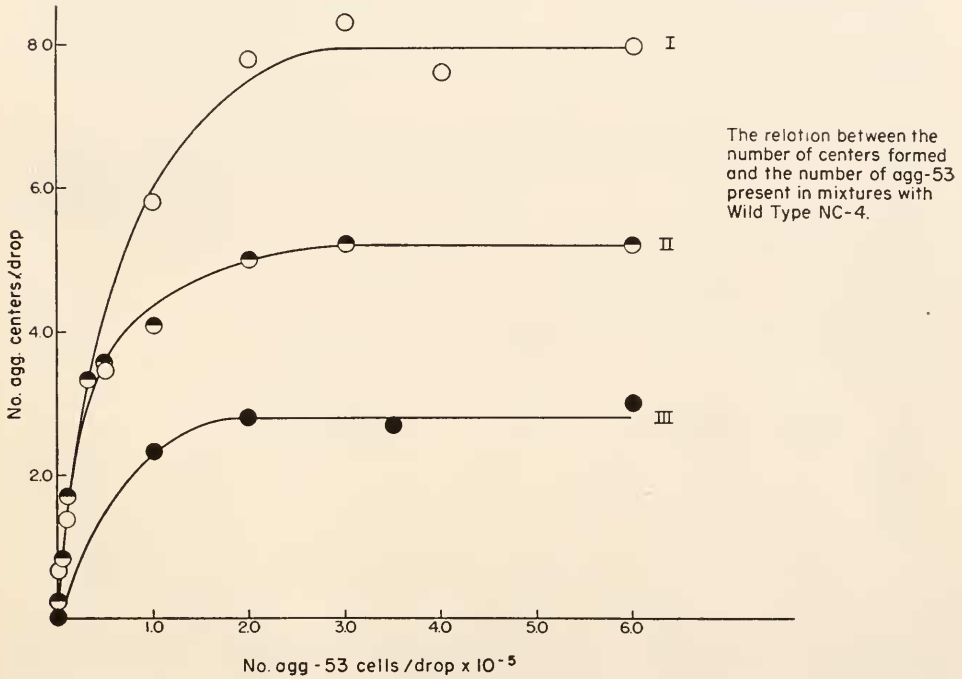

FIGURE 1. Curve I refers to mixtures containing 8.0×10^3 wild type cells/sample with various numbers of agg-53 cells. Curve II refers to mixtures containing 5.0×10^3 wild type/sample and Curve III, 2.6×10^3.

minimal agar, 10 drops to a plate and two or three plates for each mixture. Thus, the number of Nc-4/drop was 5.0×10^3 while the numbers of agg-53/drop varied between 5×10^3 and 6.0×10^5. Drops were also dispensed from control suspensions containing either Nc-4 or agg-53 in concentrations comparable to those in the experimental suspensions.

After distribution of the drops, the plates were allowed to stand with covers ajar until the drops had dried. The plates were incubated at 22° C. and twice during the following 48 hours the drops were examined either under 20 × or 100 × magnification in order to count the aggregates or fruiting bodies derived therefrom.

Thirteen of these determinations were made using seven different quantities of Nc-4 cells/drop ranging from 1.0×10^3 to 1.0×10^4. Figure 1 shows the results of three such experiments. Each point in the curves represents the mean of 30 replicate drops.

In the absence of agg-53 cells, 2.6×10^3 Nc-4 produced no centers (curve III of Fig. 1), as a result of the extremely low population density. (From the average radius of the drops and the number of cells deposited, the density was estimated to be less than 50 cells/mm.^{2}) Alone, 5.0×10^3 Nc-4 formed a mean of 0.21 centers/drop; 8.0×10^3 Nc-4 produced 0.7 centers/drop. Control suspensions of agg-53 cells were never observed to give rise to aggregate centers. Addition of

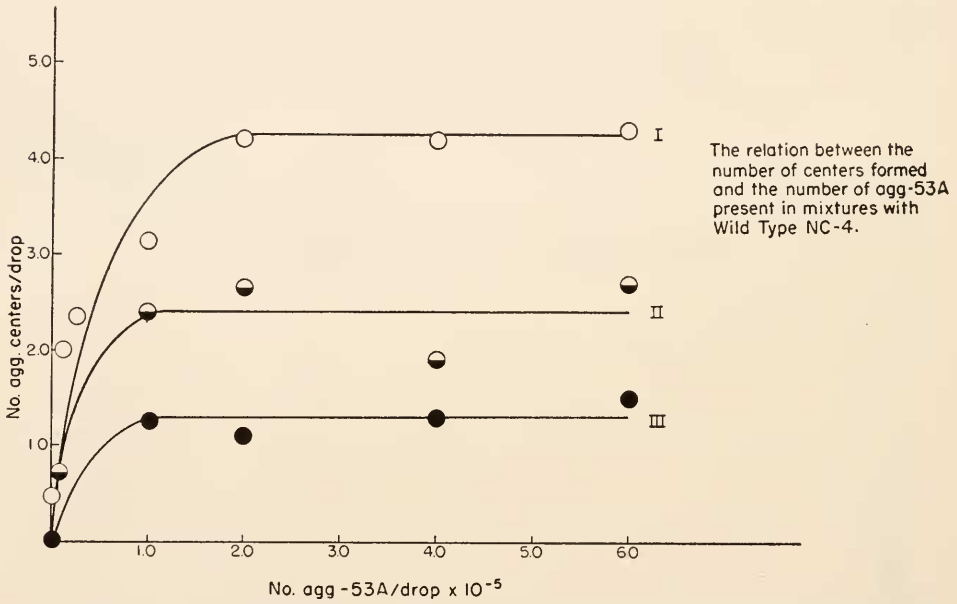

FIGURE 2. Curve I refers to mixtures containing 8.0×10^3 wild type cells/sample with various numbers of agg-53A. Curve II refers to mixtures containing 5.0×10^3 wild type cells/sample and Curve III, 2.0×10^3.

agg-53 cells to the wild type suspensions, however, markedly increased the numbers of centers formed. The presence of between 2 and 3×10^5 agg-53 cells resulted in maximal center formation whose absolute value depended upon the number of wild type. Thus for 8×10^3 Nc-4, a plateau value of 7.9 centers/drop was reached. For 5×10^3 Nc-4 the value was 4.9 and for 2.6×10^3 Nc-4 it was 2.8. One center was produced for approximately 1000 wild type cells present.

A similar set of experiments was performed using mixtures of Nc-4 and agg-53A. Figure 2 summarizes the results of three determinations. The curves are in qualitative agreement with those of Figure 1 except that significantly different plateaus were reached for the same numbers of Nc-4 employed. Using agg-53A as the test system, one center was formed for approximately every 1700 Nc-4 added.

C. The number of cells required to initiate the formation of an aggregative center

It has been mentioned that the results of a previous investigation indicate that only a small proportion of the myxamoeboid population possesses the ability to initiate center formation under the conditions of the experiment. The question of how many cells were required to initiate a center could not be answered conclusively from the data although a number of arguments could be raised to support the interpretation that a single cell can evoke the aggregative process. The inconclusiveness proceeded from the fact that in a population of wild type cells, one is concerned not only with the ability of a small proportion to initiate aggregation but also with

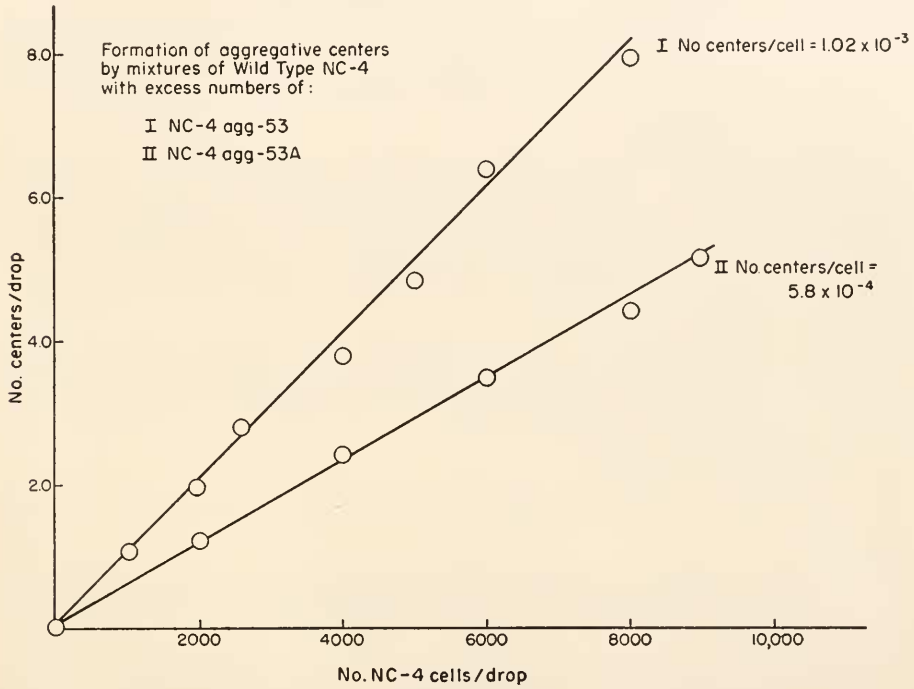

FIGURE 3. See text for experimental details.

the ability of the remaining individuals to respond to the initiating stimulus. From examination of the dependence of center formation on population number and density, it became clear that both abilities played decisive roles in the process. Thus, one could not, with this system, hope to dissociate the one from the other.

The use of aggregateless variants as the test system was considered to be capable of achieving the desired dissociation since with this system it became possible to alter the number of responding cells to any extent without changing the number of wild type individuals and therefore the number of initiator cells. Conversely, the presence of excess aggregateless cells would ensure that neither the number nor density of responding cells would limit the formation of centers. In this event, alteration of the number of wild type cells could only affect the number of initiators and it became possible therefore to examine initiating ability, *per se*.

Where the responding cells could not limit center formation, the initiation of centers by single cells would be evidenced by the fact that the number of centers formed would be directly proportional to the number of initiator cells present. For mixtures of Nc-4 and excess aggregateless variants, this would mean that the number of centers would, in fact, be proportional to the number of wild type, *i.e.,* the ratio of number of centers/wild type cell would remain constant regardless of the absolute number of wild type present. If, however, two or more cells must collaborate to evoke aggregation, the number of centers would in general vary exponentially with the number of wild type present and the ratio, number of centers/wild type cell, would approach the limit zero at infinite dilution.

A distinction between these possibilities could be made by plotting the plateau values obtained from the experiments described in section B against the numbers of Nc-4 present. It will be recalled that the plateau values represent the number of centers formed by Nc-4 initiators in the presence of excess aggregateless cells. Figure 3 shows the results.

Curve I of Figure 3 was obtained for mixtures of Nc-4 and agg-53, curve II for Nc-4 and agg-53A. Both are straight lines, indicating that with either test system, the number of centers formed is proportional to the number of wild type present, *i.e.,* the ratio of centers/wild type cell remains constant regardless of dilution. It must be concluded that for *D. discoideum,* at least, single cells do act as aggregation initiators.

In mixtures with agg-53 the mean of the centers/Nc-4 cell was calculated to be 1.02×10^{-3}, which corresponds to a distribution of one initiator cell per 980 individuals. For mixtures with agg-53A the mean was 5.8×10^{-4}, equivalent to a distribution of one initiator per 1720 individuals. The significance of this discrepancy will be discussed subsequently.

D. Proof that aggregateless cells enter into the aggregations evoked by wild type initiators

The fact that mixtures of Nc-4 and the aggregateless variants form centers whereas neither can do so alone under the conditions of test may be explained by at least four suppositions.

(a) The entire population proliferates on the minimal agar plates. Each of the cells originally laid down therefore produces a microclone. The Nc-4 cells increase in number to the extent that some can aggregate without assistance from the aggregateless individuals.

(b) The total population remains relatively constant on the minimal agar. However, the wild type cells increase in number at the expense of the aggregateless individuals. This increase might be due to growth or conceivably to the transformation of aggregateless variants into wild type. In either case the proportion of Nc-4 in the population would rise. The net increase would be sufficient for them to aggregate without assistance from the agg-53 cells.

(c) The numbers of both Nc-4 and agg-53 cells remain constant. The aggregateless cells act as "conductors" of the stimulus imposed by initiator cells in the wild type population. This conductance enables the responding cells of the wild type population to aggregate without, however, the active participation of the aggregateless individuals in the aggregation.

(d) The aggregateless cells, like the responding cells of the wild type population, are affected by the initiating stimulus and enter into the aggregate eventually formed.

It was possible to test the validity of the first two suppositions by making differential and total counts of the mixed population on the minimal agar plates at intervals between the time of deposition and the onset of aggregation. Were assumption (a) valid, the total population would increase significantly. Were assumption (b) valid, the proportion of Nc-4 in the population would increase significantly.

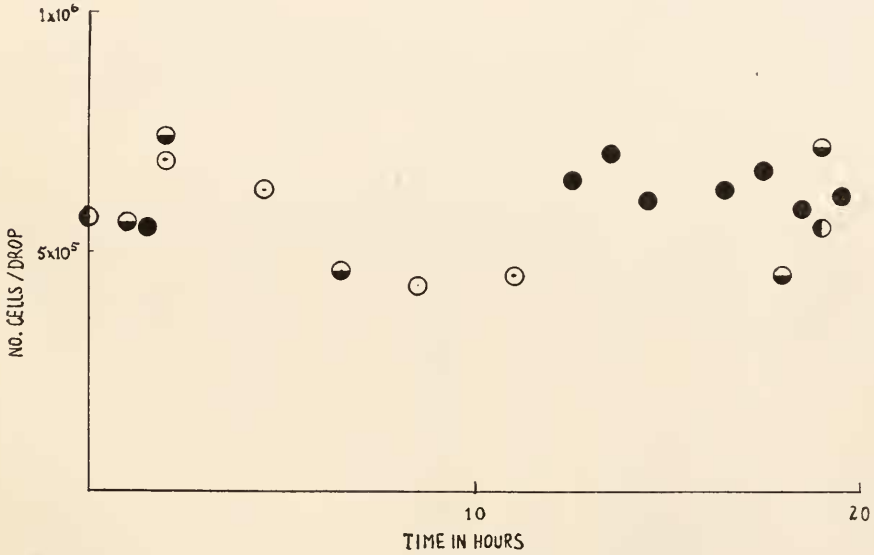

FIGURE 4. Total viable population counts of cell mixtures between the time of deposition and the onset of aggregation. See text for details.

The deposition of drops containing mixtures of Nc-4 and agg-53 on minimal agar plates was accomplished in the usual manner. Two mixtures were used: 10% Nc-4 cells (I) and 1.5% Nc-4 (II). The latter value falls within the limits employed in the previous experiments. The drops were allowed to dry and the plates were incubated at 22° C.

To obtain quantitative recovery of the cells, a flamed cork borer was used to cut plugs in the agar, each plug encompassing the area of a drop. Each plug was then suspended in 5.0 cc. salt solution and thoroughly triturated. Control experiments indicated that between 90 and 100% of the cells may be recovered from the plugs in this manner. Direct counts were made on each of the suspensions and appropriate dilutions were plated in quadruplicate with *A. aerogenes* according to the procedure given in the Methods section for clonally isolated platings. After incubation, total and differential plaque counts were made.

The plugs were collected at intervals until the beginning of aggregation. The drops prepared from mixture I showed evidence of aggregation at 12–14 hours. The cells in mixture II aggregated at 20–23 hours. Samples could not be taken

after these times because of excessive clumping of the cells and adherence to the agar.

Figure 4 illustrates the total population counts from four experiments. It will be noted that the viable counts cover the entire span between the deposition of the cells and the onset of aggregation. Each point represents the mean of counts obtained from four to eight plugs (16 to 32 plates). It is clear that no significant increase in cell number occurred on the aggregation plates. The mean number of plaques/plate was 59.2 with a variance of 109. The direct cell counts remained constant as well. The fact that extensive proliferation does not occur makes supposition (a) untenable.

Table I summarizes the results obtained from differential plaque counts. Neither mixture I nor II showed a significant change in the proportion of Nc-4 cells between zero time and the onset of aggregation. It must be concluded that assumption (b) is invalid.

TABLE I

The proportion of wild type cells in the mixed populations during incubation on minimal agar

Mixture	Time in hours after deposition	Total no. plaques	No. wild type plaques	% Wild type
I	2	1103	101	9.2
	4.5	1198	93	7.8
	8.5	347	31	9.0
	11	665	54	8.1
II	1–2	1019	15	1.5
	4.5	1096	16	1.5
	8.5	340	5	1.5
	11 –13.5	949	16	1.7
	14.5–16.5	501	10	2.0
	17.5–23.5	2095	34	1.6

Replicate population samples were collected at the stated intervals, suspended and plated. After incubation the plaques were surveyed and the proportions of wild type clones were determined.

Microscopic observation of aggregations of mixed populations has made supposition (c) highly unlikely. When the number of aggregateless cells is not overwhelmingly large, it may be seen that the entire area immediately surrounding the completed aggregate has been cleared of cells. Were the aggregateless individuals serving merely as conductors of the stimulus and not entering the aggregates, it would be impossible for every cell near the aggregate to have been removed since the ratio of aggregateless cells to wild type in such mixtures can be as high as 50:1. Moreover, visual comparison of aggregative patterns produced by control suspensions of the wild type alone and in conjunction with aggregateless cells revealed that the latter were far more extensive than the former.

In the absence of any other interpretations, one must conclude that the aggregateless cells do indeed enter into the aggregations evoked by wild type initiators. In this connection, the results of Pfützner-Eckert (1950) are of interest. Myx-

amoebae of the strain incapable of fruiting were shown to orient around and stream toward wild type pseudoplasmodia which had been placed in their midst.

E. The phenotypic composition of the sori from mixtures of wild type and agg-53

Mixtures of wild type and agg-53 were prepared in the usual manner and dispensed on minimal agar. The following aliquot volumes and proportions were employed:

Mixture	Vol. of aliquots delivered	No. Nc-4	No. Agg-53	Ratio Nc-4:Agg-53
1	0.01 cc.	6.0×10^3	3.0×10^5	1:50
2	0.02 cc.	6.0×10^3	5.0×10^5	1:83
3	0.04 cc.	6.0×10^3	8.4×10^5	1:139
4	0.08 cc.	6.0×10^3	1.4×10^6	1:232

In this manner a constant number of wild type cells was distributed over different surface areas while the variation in numbers of agg-53 provided constant population density. The particular concentrations of agg-53 were chosen on the basis of experiments (Sussman and Noël, 1952) from which the relation between the volume of aliquot and the surface area covered had been determined.

After the plates had been incubated for 36 hours, approximately 6 aggregative centers/aliquot had been formed in each of the mixtures. Single spore masses were picked and plated with bacteria. After four days incubation, the plaques were examined. The approximate numbers of plaques surveyed for the four mixtures cited above were, respectively, 1430, 1000, 350 and 204. In every case, not a single agg-53 plaque was observed. All produced normal fruiting bodies.

These results would appear to indicate that aggregateless cells do not enter into the aggregates initiated by the wild type. However, in view of the results described in the previous section, this is not considered to be a likely possibility. Two additional explanations can be offered. First, it may be presumed that only the wild type cells within the mixed aggregates eventually become spores. Second, one might suppose that any aggregateless cells which enter the aggregates either spontaneously revert or are transformed to wild type. The implications which the latter possibility possesses both for the genetic nature of the aggregateless variant and for cellular interactions during the developmental process would seem to demand that a distinction between these interpretations be attempted.

Discussion

The information at present available makes it possible to formulate a description, albeit tentative and incomplete, of the myxamoeboid population undergoing aggregation. During the stationary growth phase, the wild type population may be divided into at least two phenotypic classes. These are the initiator cells, each of which can evoke the formation of an aggregative center, and the responding cells, which can proceed to aggregate only upon reception of the stimulus imposed by an initiator cell.

Two independently derived sets of data indicate that only a small proportion of the cells possesses initiating capacity under the conditions of test. The precise proportion of initiators in *D. discoideum* is in doubt. An examination of center

formation by individuals of strain Nc-4 had previously indicated that there exists one initiator cell per 2110 myxamoebae (Sussman and Noël, 1952). But the results obtained from mixtures of wild type with Nc-4 agg-53A indicate a ratio of one initiator per 1720 cells and a corresponding analysis employing Nc-4 agg-53 as the test system suggests a value of one initiator per 980 cells. The discrepancy between the first two values is probably significant, that between the first or second and the last is certainly so.

A tentative explanation of these differences, which we favor currently as a working hypothesis, rests upon two assumptions: (a) There is a distribution of initiating ability in the wild type population, a few cells possessing a very high order, others inferior orders. These inequalities may stem from differences in a particular biosynthetic capacity which is associated with the process of initiation. (b) The ability of cells to respond to the initiating stimulus is a function of experimental conditions such as population density and depends also upon the strain of organisms employed.

From these suppositions, one might expect that for responding cells which possess a low level of sensitivity, only a portion of the potential initiators might have acquired sufficient initiating capacity to evoke the aggregative process. The greater the sensitivity of the responding cells, the higher would be the proportion of individuals which could be classed as initiators. In fact, were responding cells available which possessed extreme sensitivity, quite possibly the entire wild type population might be shown to have attained some degree of initiating ability.

On this basis, the previously cited discrepancies become understandable. When wild type individuals are mixed with aggregateless variants, the proportion of initiators will depend upon the sensitivity of the particular variant employed, hence the difference in the results obtained with agg-53 and agg-53A. Given aggregateless cells whose sensitivity approaches that of the responding cells in the wild type population, the proportion of initiators which appears in the mixture would be higher than when the wild type itself serves as the test system, even when the latter is at optimal population density. This stems from the fact that the density of responding cells in the former case can be made enormous and consequently even relatively small initiating capacities of some wild type cells would evoke a response. When wild type is employed alone, however, this concentration of responding cells cannot be attained. If one were to move the responding cells closer together, one would be simultaneously concentrating the initiator cells. The closer the proximity of these cells, the greater would be the opportunity for them to compete for responding cells, with the inevitable result that the maximal number of centers of which the population is capable would not appear and the apparent proportion of initiators would be decreased. Experimental evidence (Sussman and Noël, 1952) indicates that at population densities greater than 200–300 cells/mm.^{2} the decrease becomes significant and progressively larger. But this density is two orders of magnitude below that which can be achieved by addition of aggregateless cells.

While this is by no means the only interpretation which can be drawn, it offers readily testable inferences that are under examination at present. Its value lies in the fact that it offers an explanation of the genetic mechanism controlling the observed expression of cell heterogeneity. It suggests that the differences between initiator and responding cells may not be genic in nature but rather expressions of enzymatic adaptation against a constant genic background. That phenotypic altera-

tions involving relatively small segments of a population can be ascribed to non-genic variations in enzyme-forming capacity has been demonstrated in connection with the phenomenon of long-term adaptation in yeast (Spiegelman, Sussman and Pinska, 1950).

It is a pleasure to acknowledge the valuable assistance of Mrs. Min Zakarian in the performance of the experiments reported here.

Summary

1. A study has been made of the formation of aggregative centers by mixed populations of *D. discoideum* wild type and aggregateless variants. The latter are capable of responding to the aggregative stimulus but not of producing it. The following results were obtained:

(a) Under conditions where the wild type is too dispersed to aggregate alone, addition of aggregateless cells permits the formation of aggregative centers. When the number of centers so formed is plotted against the number of variant cells present, a saturation curve is obtained.
(b) For the case where the aggregateless cells are present in excess, the number of centers formed is directly proportional to the number of wild type added.
(c) Neither the total population nor the proportion of wild type cells changes significantly during the period of incubation prior to the onset of aggregation.

2. These findings demonstrate that the evocation of an aggregative center is accomplished by a single individual in the wild type population. These have been termed "initiator" cells. Under the conditions employed, the remainder of the wild type individuals and the aggregateless variants are the "responding cells" which can aggregate only in the presence of an initiator.

3. Estimates made of the proportion of initiators in the wild type population, using as test systems the wild type population itself as well as two aggregateless variants, range between one per 980 and 2110 individuals. The discrepancy is considered to be real and an explanation is offered.

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